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Effect of Sublethal Doses of Botulinal Toxin on the Organism Following Multiple Administrations

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The question of the effect of sublethal doses of exotoxins on the organism has barely been clarified in the literature since BEHRING (1893) detected this paradoxical phenomenon. The nature of the phenomenon is confined to the fact that if animals receive multiple small doses of diphtheria or tetanus toxins, they acquire a high sensitivity to these poisons. At the same time, antitoxin in animals' blood may occur in a quantity that is fully sufficient to neutralize many fatal doses of the toxin involved. Multiple administrations of small doses of toxin cause usual clinical manifestations that are characteristic of reactions to exotoxins.

BEHRING, explaining this phenomenon stated that, during immunization process by toxins, animals acquire not only immunity, but increased sensitivity as well. According to N.F.GAMALEI (1939), after injection of multiple small doses of toxin, the resultant death of animals is explained by the impairment of resistivity in

cells invaded [redacted] by the toxin and, perhaps, also in the central nervous system. It seems to us that the latter explanation is most accurate, inasmuch as only neurotrophic toxins possess the characteristics that tend to induce the BEHRING phenomenon.

In 1936, when MINKEVICH and KOTLYAROVSKAYA administered sublethal doses of toxin in order to develop experimental botulism in animals that were infected with spores, they disclosed a greater significance of this factor in the pathogenesis of botulism. We determined (1947) that animals perished due to botulism after they received multiple sublethal doses of the toxin.

The development of increased sensitivity to small doses of bacterial toxins plays an important role in the pathogenesis of toxinfections. Pathogenic causes of this phenomenon were studied in experiments with diphtheria toxins (KRAVCHENKO and GALANOVA, 1948; MATVEEV and BULATOVA, GINDIN, 1949; KOLESNIKOVA and MATVEEV, 1951) and with tetanus toxins (MATVEEV and BULATOVA, 1950*); MORGUNOV and KHATUNTSEV, 1955).

[redacted] (1950) explained the phenomenon of increased sensitivity by summation of stimulations after multiple administrations of sublethal doses of toxins. MORGUNOV and KHATUNTSEV (1954) explained by botulinal toxins of the A and B types the immunologic specificity associated with this paradoxical sensitivity

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*) - See P.F. ZDRODOVSKI'S book: "Problem of reactivity in studies of infection and immunity", 1950, p.93.

phenomenon; they also came to the conclusion that the summation of stimulations rests at the foundation of this phenomenon.

In connection with the fact that the problem of the effect of sublethal doses of botulinal toxins on organism (following multiple administrations) still remains insufficiently clarified, we made comparative studies of the EHRING phenomenon using three types of animals.

The first series of experiments were carried out on guinea pigs. At first, we pretitrated a lethal dose for a guinea pig (hypodermic administration); it contained a dry botulinal toxin of the A type previously precipitated with ammonium sulfate. We took measures with the precipitation of the toxin to decrease the admixture of impurities occurring in the medium. The established fatal dose was diluted with a physiological solution 50, 100, 500, 1,000 and 3,000 times. Guinea pigs received daily 1/50, 1/100, 1/500, 1/1,000 or 1/3,000 of a dose. From 3 to 10 injections were necessary (see Table 1) in order to develop the symptoms of botulism in guinea pigs. The disease followed after 3 to 10 days, when the quantity of the administered toxin comprised altogether 6/50, 8/50, 7/100, 10/100, or even 4/500, 5/1,000 and 4/3,000 of the fatal dose. At the same time, a typical clinical picture of botulism developed gradually: dyspnea appeared, then weakening of the musculature (muscles became paste-like) and, occasionally, paresis was observed. Guinea pigs died from botulism on the 2d to 29th day of the sickness. In the course of three experiments we performed a treatment of animals with specific serum, but since we initiated this late,

Toxin Administered to Guinea Pigs

Pigs Falling on Various Days					Clinical Aspects	Serotherapy	Number of guinea pigs dead from botulism	Date of death of guinea pigs after illness; days	Neutralization reaction with serum of guinea pig.
6th guinea pigs	7th	7th	9th	10					
--	---	---	---	---	Dyspnea, muscles relaxed	---	4	4th to 5th	
--	---	---	---	3	The same	---	5	7th to 29th	Negative
--	---	---	---	---	The same	---	5	2nd	
6	---	1	---	1	The same and paresis of hind limbs	---	4	13th to 20th	Negative
10	---	---	---	---	Dyspnea, muscles relaxed	5 ml of serum to 3 guinea pigs each	10	7th to 13th	
--	---	---	---	---	The same	5 ml of serum each - to all	10	5th to 10th	Negative
--	---	---	---	---	The same	3 ml of serum to 10 guinea pigs each	14	2nd to 3rd	
--	---	---	---	---	The same	---	5	9th to [illegible]th	
--	---	---	---	---	The same	---	5	6th to 8th	
								62	
Control									
--	---	---	---	---	Healthy				
--	---	---	---	---	Healthy				
--	---	---	---	---	Healthy				

BEHRING'S Phenomenon of Botulin

Experiment No.	Number of guinea pigs in experiment	Single dose of toxin (Dlm)	Method of toxin administration	Numerousness of toxin administration	Quantity of administered toxin (Dlm)	Number of Guinea Ill from Botulism				
						1st	2d	3d	4th	5th
						Number of				
1	4	1/50	Daily, subcutaneously, 1/50 Dlm	7	7/50	---	---	--	---	4
2	5	1/100	Daily, subcutaneously, 1/100 Dlm	3 to 10	From 3/100 to 10/100	---	---	1	--	1
3	5	1/100	The same	3	3/100	---	---	5	--	--
4	10	1/100	The same	7	7/100	---	---	--	--	--
5	10	1/50	Daily, subcutaneously, 1/50 Dlm	6	6/50	---	---	--	--	--
6	10	1/50	4 days, morning, even., 1/50 Dlm	8	8/50	---	---	--	10	--
7	16	1/500	Daily, subcutaneously, 1/500 Dlm	4	4/500	---	---	--	16	--
8	5	1/1000	Daily, subcutaneously, 1/1000 Dlm	5	5/1000	---	---	5	--	--
9	5	1/3000	Daily, subcutaneously, 1/3000 Dlm	4	4/3000	---	--	5	--	--
Total	70									
1	7	7/50	Subcutaneously	1	7/50	---	---	--	---	--
2	10	7/100	Subcutaneously	1	7/100	---	---	--	---	--
3	8	4/500	Subcutaneously	1	7/500	---	---	--	--	--

Rabbit's No.	Date of the first administration of toxin	Daily dose of toxin (Dim)	Method of Toxin Administration
1	23 Jan.	1/100	Daily, subcutaneously, 1/100 Dim
2	23 Jan	1/100	The same
3	23 Jan	1/100	The same
4	10 Feb	1/100	The same
5	10 Feb	1/100	The same
6	10 Feb	1/100	The same
7	10 Feb	1/100	The same
8	10 Feb	1/100	The same
9	10 Feb	1/100	The same
10	10 Feb	1/100	The same
11	10 Feb	1/100	The same
12	10 Feb	1/100	The same
13	10 Feb	1/100	The same
14	10 Feb	1/100	The same
15	10 Feb	1/100	The same
16	10 Feb	1/100	The same
17	16 May	1/500	Daily, subcutaneously, 1/500 Dim
18	16 May	1/500	The same
19	16 May	1/500	The same
20	16 May	1/500	The same
1	20 Feb	8/100	Daily
2		8/100	Daily
3		8/100	Daily
4		8/100	Daily
5		8/100	Daily

Table 2

gon of Botulin Toxin Administered to Rabbits

Numerousness of toxin administration	Quantity of administered toxin	Date of falling ill from botulism	Clinical aspects of botulism in rabbits	Day of death following the beginning of the sickness	Neutralization reaction with serum and botulin toxin
7	7/100	28 Jan	Muscles relaxed, dyspnea	5th	Negative
8	8/100	30 Jan	The same	6th	Negative
11	11/100	3 Feb	Paresis of hind limbs	16th	Negative
9	9/100	19 Feb	Hind limbs' paresis, dyspnea	1st	Negative
9	9/100	19 [illegible]	The same	1st	Negative
8	8/100	18 Feb	The same	3rd	Negative
8	8/100	18 Feb	The same	4th	Negative
9	9/100	18 Feb	The same	13th	Negative
8	8/100	18 Feb	The same	1st	Negative
8	8/100	18 Feb	Muscles relaxed, dyspnea	1st	Negative
2	2/100	12 Feb	The same	7th	Negative
9	9/100	18 Feb	The same	4th	Negative
9	9/100	18 Feb	Hind limbs' paresis, dyspnea	7th	Negative
8	8/100	18 Feb	The same	3rd	Negative
9	9/100	18 Feb	The same	5th	Negative
9	9/100	18 Feb	The same	3rd	Negative
6	6/500	22 May	Muscles relaxed, dyspnea	7th	Negative
10	10/500	26 May	The same	5th	Negative
11	11/500	27 May	The same	4th	Negative
8	8/500	24 May	The same	4th	Negative
			Control		
1	9/100	Healthy	Botulin symptoms absent		
1		Healthy	The same		
1		Healthy	The same		
1		Healthy	The same		
1		Healthy	The same		

rabbits were less sensitive than guinea pigs to the action of small doses of botulinal toxin: a considerable number (39.4%) of them survived, having shown clearly expressed symptoms of botulism.

The third series of experiments were carried out on mice. In these experiments, having pretitrated previously a lethal dose of dry botulinal toxin, we diluted it with a physiological solution 10, 20, 30, 50 and 100 times, and we administered it daily, subcutaneously, to mice in quantities of 1/10, 1/20, 1/30, 1/50 and 1/100 of the lethal dose. The mice were resistant to the toxin following its multiple administration in sublethal doses. But, after three injections of the toxin in 1 Dlm quantity, all 10 mice became sick from botulism and died on the 4th to 6th day of illness. The effects of a dose equal to 1/2 Dlm caused death in 5 out 10 mice after 7 to 8 days. A daily administration of 1/30 Dlm to these animals brought almost the same results. Having administered 10 injections of 1/50 Dlm to 20 mice, 8 of them died, whereas after 12 injections of 1/100 Dlm, no visible signs of clearly manifested clinical symptoms of intoxication could be observed. In one experiment, all mice that received this dose of toxin, showed slight symptoms of botulism, but remained alive.

All experiments of the third series included 60 mice and 29 of them died from botulism. Thus, we see that more than 50% of the animals remained alive. Consequently, mice were found to be considerably more resistant to small amounts of the toxin than guinea pigs and rabbits. Analogous results were obtained in experiments on mice with toxins of the B, C and E types.

The animals also showed a developed botulism sickness after multiple peroral administrations of sublethal doses of botulinal toxins. All animals died that received per os tenths and hundredths portions of one lethal dose of the toxin.

It should be noted that after control guinea pigs, rabbits and mice received a single subcutaneous or enteral injection of a whole toxic dose that was used for a partial administration, botulism symptoms failed to develop and all animals remained alive.

We established (1947) that toxins of causative agents of gaseous gangrene, given in small doses, did not produce a development of hypersensitivity in animals. This was subsequently confirmed by MORGUNOV, KHATUNTSEV and YAGUR (1954).

OSTRYI, SOBLEVA and ALIEV (1956) proved that the process of summation of pathogenic neural sensitizations, following administration (in microintervals of time) of subliminal doses of toxins that cause gaseous gangrene, may lead the organism to a fatal outcome.

In order to clarify the effectiveness of the action of sublethal doses of other toxins, we conducted experiments with dry toxins of Cl. perfringens and Cl. oedematiens. In both instances the experiments were carried out on 15 guinea pigs. The animals received a toxin subcutaneously and daily in quantities of 1/20, 1/50 and 1/200 of Dlm for one guinea pig. We obtained negative results in all instances, even after 15 or 16 injections. Prolonged observations revealed that all animals remained alive and showed no symptoms of illness. Only in sporadic cases in guinea pigs that

received small doses of Cl. oedematiens we observed light spasms and inconsequential dyspnea, but the latter disappeared quickly. Consequently, the experiments proved that the toxins of Cl. perfringens and Cl. oedematiens failed to produce the BEHRING phenomenon in the organism after multiple injections of small doses. This indicates that the phenomenon is caused only by toxins with distinctly expressed neurotropic characteristics.

Hence, it is obvious from the performed investigations that involved the studies of the BEHRING phenomenon in connection with the botulinal toxin type A that, after a multiple administration of sublethal doses of the toxin to animals, the latter gradually developed an impairment of important and vital processes, which caused their death. We took an interest in clarifying, whether any immunity develops in animals following a multiple administration of the toxin. As we mentioned above, antitoxin was absent in the serum of guinea pigs and rabbits that received multiple small doses of the toxin: 1 ml of serum of these animals failed to neutralize even one lethal dose of the toxin for mice.

In order to determine the immunological condition of the organism of animals after multiple administration of small doses of toxin, we conducted experiments in vessels on rabbits' ears according to the method of KRAVKOV and PISEMSKI.

We carried out three groups of experiments on rabbits. The animals of the first group received subcutaneously 1/50 Dlm daily of botulinal toxin prepared in broth of oxen's meat. As soon as rabbits became sluggish, but the symptoms of botulism were not

yet clearly manifested, we discontinued injections of the toxin. Altogether, we made 5 injections and thus we administered 1/10 Dlm.

The experiments in vessels of rabbits' ears were carried out during a period from the 5th to 11th day after the first administration of toxin. The latter was prepared in broth of rabbits' meat in order to exclude a possibility of development of anaphylaxis. For experiments in vessels we used a dry toxin precipitated with ammonium sulfate; the toxin was diluted 1:100, and later dialyzed in colloidal kit, at first for 18 to 20 hours against faucet water and, subsequently, for 24 hours against distilled water. At the beginning we passed the toxin through the vessels using the dilution of 1:50,000 and, later, that of 1:25,000 and 1:10,000 (for dilution we used the RINGER-LOKK solution). Thus, to be able to clarify the actual nature of the reaction in rabbits' vessels on botulinal toxin, we passed through the vessels of one ear of animals the solution of the toxin, and adrenalin through the vessels of the other ear.

The conducted experiments provided a quite clear answer to the question about the conditions of vessels in rabbits' ears on the 5th to 11th day following the injection of toxin. It was found that the vessels in these animals contracted considerably milder (by 1.2; 0.4; 10%) on the passing of toxin's solutions, than the vessels of normal animals (by 24.4; 24.3; 25.3%). The vessels in the ears of experimental animals contracted mildly (by 9 to 14%) when the solution of adrenalin was applied, while the vessels of normal rabbits contracted very sharply (by 40.3 to 51.7%). Conse-

quently, the unresponsive condition of vessels in rabbits' ears after daily sublethal doses of the toxin depended on a prior contraction under the effects of the toxin.

The described experiments proved how profound were the changes that took place in the organism of animals following the administration of very small doses of the toxin. The process was accompanied not only by disorders in myogenic tonus and myoneural connections, but also by a considerable change in tonus of the vascular system.

In the second group of experiments 9 rabbits received daily 1/100 Dlm of the toxin each until the botulism symptoms appeared in animals. After 7 to 11 injections of the toxin, only 5 rabbits survived out of 9. The surviving 5 rabbits, having recovered within 38 to 40 days, were subjected immediately to an experiment in vessels of animals' ears. The results of these experiments appeared to be completely different from those obtained with the first group of animals. The vessels in rabbits' ears reacted considerably milder on passing of solution of the toxin; at the same time, they reacted on solution of adrenalin almost like the vessels in normal animals. One can conclude from this that, as a result of injections of small doses of toxin to rabbits, the latter showed, at first, a sharp contraction of vessels, but, after a prolonged time, the cells developed an immunity to botulinal toxin in smooth musculature of the vessels.

Because the quoted data were obtained with participation of a limited number of involved animals, we decided to begin the third

group of experiments, using 13 rabbits. In these experiments we used animals, which recovered from botulism after they received sublethal doses of the toxin in order to produce the BEHRING phenomenon (see Table 2). All animals, that received 8 to 11 injections of 1/100 Dlm of the toxin, became sick from botulism and 20 rabbits died out of 33. Then, 26 to 46 days later, we conducted experiments in vessels of animals that survived the first injection of the toxin; the experiments were carried out according to the method of KRAVKOV and PISEMSKI. We passed a solution of toxin through vessels of one ear and a solution of adrenalin through vessels of another ear. The results proved to be analogous to the previous results: the vessels in the ears of rabbits that survived multiple injections of small doses of botulism reacted on dilutions of this toxin less acutely than the vessels of normal animals. The contraction of vessels in rabbits, that received sublethal doses of the toxin for a prolonged time, reached 10, 10.5 and 13.8%, while in animals that received adrenalin, the precentage was 38, and from 37 to 52. The contraction of vessels in normal rabbits on the same doses of toxin was 20.7, 21.7 and 24.3%, i.e. it was two times stronger; then, the 32.3 to 48.2% contraction on adrenalin was almost the same as that in animals which received the toxin.

The discussed results offer a reason to a claim that, after some time following administration of small doses of toxin, immunity develops in rabbits in the cells of smooth musculature in vessels. The condition of increased sensitivity to the toxin (sharp contraction of vessels) that prevails at first, changes subse-

quently to immunity of vessels to botulinal toxin. At the same time, the accumulation of antitoxin in the blood in a quantity sufficient for detection with the aid of neutralization reaction - does not take place. In this case, the immunity of tissues was not dependent on the presence of antibodies.

It is obvious from our investigations that botulinal toxins, administered in multiple and very small doses, can injure an organism and cause its destruction. This was also verified by GUYTON and McDONALD (1947); their data indicate that, 0.1 of one molecule of the toxin type A, applied to one neural end plate, is sufficient to cause histological changes there. At the same time, no disorders in neural conductivity occur, however, in the myoneural connection, an important and vital process is disturbed, which is connected with the production of acetylcholine.

Following a multiple administration of botulinal toxin in insignificant doses to animals, the latter may suffer profound disorders in important and vital processes of the central and peripheral nervous system, as well as in other tissues, and this causes death of animals. All this suggests a supposition that the toxin is an antimetabolite; while acting on the organism, it impairs the processes of metabolism, consequently the physiological function of cells and tissues is disturbed in the organism. Therefore, after a multiple administration of sublethal doses of toxins in our experiments, a summation of injuries and destructions resulted in myoneural connections and in other physiological processes of the organism, but not a summation of stimulations. With an increased

dose of toxin, injuries in the organism of animals developed with a particular rapidity. Thus, the hypersensitivity from a multiple administration of small doses of toxins evoked all resultant enlarged injuries in tissues of the organism.

The results obtained by us are important to interpretation of the pathogenesis of intoxication from food poisonings by Cl. botulinum. It is obvious from the conducted investigations that, to cause a sickness in man and animals, a multiple poisoning with sublethal doses of botulism is sufficient.

In food poisonings with a short incubation period, the first larger dose of toxin ingested with foodstuffs into the organism causes a grave affliction and, at the same time, a high hypersensitivity in the organism of a patient develops to new doses of the toxin that are produced by the microbe. In such instances, the observed brief incubation period not always permits a clarification of the role of toxin that is developed in the organism.

In instances of poisoning, when the first dose of toxin is ingested with foodstuffs is small, the incubation period lasts several days. During this time, the microbe produces new doses of toxin in the organism of man and animals; these doses gradually affect the organism and thus botulism develops that, in some cases, results in death of a patient.

The experiments carried out by us indicate that, in such cases, insignificant quantities of toxin, produced in the organism of a patient, are sufficient to develop the disease.

Practically speaking, instances were also observed in food

poisonings that the botulism disease may develop in people and in animals with a multiple ingestion of any product. In such cases, the toxin in a product was in small quantities, frequently not detectable with the aid of biological test, or neutralisation reaction on mice, or guinea pigs. Nevertheless, after a multiple admittance of small quantities of toxin and when a microbe is ingested with foodstuffs, botulism sickness develops in the organism of man or animals as a result of the evoked BEHRING phenomenon. Such cases are observed in people who use for a daily food the same brand of ham, or salty red herring and other products of home canning.

Conclusions

1. Following a multiple administration of sublethal doses of Cl. botulinum toxin to guinea pigs, rabbits and mice, botulism disease and death resulted, although the aggregate dose of administered doses was considerably lower than a fatal dose. With a single administration of the entire quantity of toxin that was used for multiple injections, animals involved remained healthy.

2. Guinea pigs were the most sensitive to multiple administrations of sublethal doses of the A toxin; rabbits and mice were less sensitive.

3. After administration of small doses of toxin, the vessels of animals acquired hypersensitivity at first and immunity later.

4. A multiple effect on organism caused by sublethal doses of toxin plays an important role in the pathogenesis of botulism.

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Summary (copied)

The Cl. botulinum toxin administered repeatedly to guinea pigs, rabbits and mice provokes botulism and death with the ~~sum~~ total dose of the toxin administered being much below the lethal one. With a single administration of the total dose, the animals remained unaffected. Guinea pigs were more sensitive to manifold administration of botulinus A toxin in sublethal doses than rabbits and mice. When introducing small doses of the toxin, the vessels first showed an increased sensitivity with their immunity developing at a somewhat

later date. Manifold administration of botulinus toxin in sublethal doses plays an important role in the pathogenesis of botulism.